IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial No. : 10/577,982

Applicant : Makoto KOIZUMI

Filed : May 2, 2006

For : OLIGONUCLEOTIDES HAVING A 2'-0,4'-C-ETHYLENE NUCLEOTIDE IN THE THIRD POSITION

Art Unit : 1637

Examiner : Mark STAPLES

Docket No. : 06189/HG

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DECLARATION UNDER 37 CFR 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

MAIL STOP RCE

S I R :

I, Makoto Koizumi, the inventor of the above-identified application, declare as follows:

A. My Education and Experience

1. I graduated from Hokkaido University, Sapporo, Japan, in the year 1986, and I received a doctorate degree from Hokkaido University in the study of catalytic ribozymes with sequence-specific RNA cleaving activity in 1991.

2. I have worked for Sankyo Company, Limited, Tokyo, Japan, since 1991. My research activities at Sankyo Company, Limited have included the following:

synthesis of nucleoside analogs with antibiotic activity and synthesis of modified oligonucleotides with antivirus, anticancer and anti-diabetes activity. My research activities were not limited to those in the company; I studied as a visiting researcher at Yale University, New Haven, CT, for two years from November 1997. I presently hold the position of the Chief Researcher of Core Technology Research Laboratories of Sankyo Company, Limited
I have held the position of the Senior Researcher of the Exploratory Research Laboratories of Daiichi Sankyo Company, Limited since the year 2007.

3. I am a member of the Pharmaceutical Society of Japan. I am on the committee of Antisense DNA/RNA Society, Japan.

4. I have contributed many scientific papers. For example, I am a co-author of "Biologically active oligodeoxyribo-nucleotides. 5. 5'-End-substituted d(TGGGAG) possesses anti-human immunodeficiency virus type 1 activity by forming a G-quadruplex structure." *J. Med. Chem.* 1998, 41, 3655-3663; "Allosteric selection of ribozymes that respond to the second messengers cGMP and cAMP." *Nat. Struct. Biol.* 1999, 6, 1062-1071. Also, I am a corresponding author of "Synthesis and properties of 2'-O,4'-C-ethylene-bridged nucleic acids (ENA) as effective antisense oligonucleotides." *Bioorg. Med. Chem.* 2003, 11, 2211-2226; "Triplex formation with 2'-O,4'-C-ethylene-bridged nucleic acids (ENA) having C3'-endo conformation at physiological pH." *Nucleic Acids Res.* 2003, 31, 3267-3273; "Direct comparison of in vivo antisense activity of ENA oligonucleotides targeting PTP1B mRNA with that of 2'-O-(2-methoxy)ethyl-modified oligonucleotides." *Oligonucleotides* 2006, 16, 253-262.

5. I am named as an inventor of many patents issued in Japan, the United States and other countries. I am listed as an inventor in the following US patents: "Looped hairpin ribozyme." U.S. Patent 5,631,115 (issued May 20, 1997); "Modified oligodeoxyribonucleoditides." U.S. Patent 5,674,856 (issued October 7, 1997); "Composition and method for the treatment or prophylaxis of viral infections using modified oligodeoxyribonucleotides." U.S. Patent 5,807,837 (issued September 15,

1998).

6. I was the recipient of the following academic and professional awards: "JB award, the Japanese Biochemical Society, 1996"; "Bioorganic Medicinal Chemistry Most Cited Paper 2003-2006 Award, 2006".

7. I am an expert in the way and manner of selecting and synthesizing compounds suitable for development as pharmaceuticals based on the chemistry and biology of nucleosides, nucleotides and oligonucleotides.

B. I am providing my opinion herein concerning the level of skill of a person of ordinary skill in the art of designing and synthesizing pharmaceutical compounds.

1. A person of ordinary skill in the art is a hypothetical person who is presumed to be aware of all the pertinent prior art.

2. A person of ordinary skill in the art is a person to whom an expert in the art could assign a task of moderate difficulty, with reasonable assurance that the task would be accomplished without a great deal of supervision.

3. The following factors should be considered in determining who is a person of ordinary skill in the art: the types of problems encountered in the art; the prior art solutions to those problems; the rapidity with which innovations are made; the sophistication of the technology; and the educational level and experience of active workers in the field.

4. A person with a graduate degree, such as a Master's Degree or a Doctorate Degree, in a field requiring knowledge of organic chemistry and/or biochemistry. The person with a Master's Degree should have at least five years research experience. The person with a Doctorate Degree should have at least two years of post-graduate academic or two years of post-doctoral research in a company. The research experience of such a person should be in working for a pharmaceutical company in the development of pharmaceuticals to achieve related pharmaceuticals or medical organic compounds having the same or enhanced properties.

C. The following statements of fact and opinion hereinafter, in my opinion, would be the statements of fact and opinion of a person of ordinary skill in the art identified hereinbefore.

1. Latorra et al., Human Mutations, (2003), 22, 79-85 (hereinafter referred to as "Latorra et al.") was cited as the primary reference in three rejections under 35 USC 103 in the August 20, 2008 Office Action in the above-identified application. A secondary reference in all of said three rejections under 35 USC 103 was a publication of which I am a coauthor, namely, Koizumi et al., Nucleic Acids Research, (2003), 13, No. 12, 3267-3273 (hereinafter referred to as the "Koizumi et al. publication").

2. The left and right columns of page 80 of Latorra et al. refer to a locked nucleic acid (LNA) fixed at the 3' terminal position.

3. On page 4 of the aforesaid August 20, 2008 Office Action, reference was made to the legend of Table 1 on page 81 of Latorra et al., wherein the following is stated:

> "A total of 16 forward DNA and 3' LNA primers were designed for each of three pUC19 targets, and included match and the three other possible mismatch combinations at the last four positions of each 3' end."

4. It is respectfully submitted that a person of ordinary skill in the art would not consider that the above sentence teaches placing a LNA at the third position from the 3' end for the following reasons:

Solid-phase synthesis of oligonucleotides by using controlled pore glass (CPG) is described on page 117, lines 13 to 16 of Nucleic Acids in Chemistry and Biology, edited by G. Michael Blackburn and Michael J. Gait, as follows:

> "The 3'-terminal deoxynucleoside of oligonucleotide to be synthesized is attached to the CPG support by conversion of its 5'-O-DMTr-(N-acylated)-derivative into the corresponding 3'-O-(4-nitrophenyl) succinate, which is subsequently reacted with the amino groups on the support (Fig. 3.59)."

In the oligonucleotide solid-phase synthesis, the CPG, which is attached with the 3'-terminal nucleoside of oligonucleotide to be synthesized, is used as a solid support. If a person of ordinary skill in the art would consider to synthesize DNA Forward primer 2 5'- GCGAAAGGGGGATGTGCTGCA -3' in Table 1 on page 81 of Latorra et al., the CPG, which is attached with the 3'-terminal A, such as "A-succinate-CPG", would be used as the solid support.

In addition, attention is directed to the general principles of assembly of oligonucleotides chains using phosphoramidite chemistry as described on page 117, line 6 from the bottom to page 119, line 14 of Nucleic Acids in Chemistry and Biology, edited by G. Michael Blackburn and Michael J. Gait (copies of said pages 117 to 119 of this publication are submitted concomitantly herewith).

In the second step, if a person of ordinary skill in the art would consider synthesizing the aforementioned DNA Forward primer 2, 5'-GCGAAAGGGGGATGTGCTGCA-3', a person of ordinary skill in the art would

elongate the oligonucleotide chain to "A-succinate-CPG" using appropriate DNA phosphoramidites to obtain "5'-GCGAAAGGGGGATGTGCTGCA- succinate-CPG".

According to deprotection and removal of the oligonucleotide from the support described on page 119, line 11 of Nucleic Acids in Chemistry and Biology, edited by G. Michael Blackburn and Michael J. Gait, the succinate-linkage is cleaved with a mild aqueous base.

In the final step, if the person of ordinary skill in the art would consider to synthesize the aforementioned DNA Forward primer 2, 5'-GCGAAAGGGGGATGTGCTGCA -3', a person of ordinary skill in the art would treat 5'-GCGAAAGGGGGATGTGCTGCA -succinate-CPG with a mild aqueous base to obtain the aforementioned DNA Forward primer 2, 5'-GCGAAAGGGGGATGTGCTGCA -3'.

On the other hand, in the left column on page 81, lines 3 to 5 of Latorra et al., the following is stated for a LNA primer synthesis:

> "PCR primers were synthesized using standard phosphoramidite chemistry by Proligo LLC (Boulder, Co; www.proligo.com). The 3'LNA residues were attached to controlled pore glass."

In order to synthesize LNA Forward primer 2, 5'-GCGAAAGGGGGATGTGCTGCA-3', containing the LNA residue, a person of ordinary skill in the art would consider that Latorra et al. used CPG, which is attached to the 3'-terminal LNA-A, "(LNA-A)-succinate-CPG", as the solid support.

In addition, in the left column on page 81, lines 5 to 7 of Latorra et al., the following is stated for the elongation of the remaining sequences:

> "The remaining sequences were made using standard DNA phosphoramidites produced by Proligo LLC."

In order to synthesize LNA Forward primer 2, 5'-GCGAAAGGGGGATGTGCTGCA-3', containing the LNA residue, a person of ordinary skill in the art would consider that Latorra et al. elongated the oligonucleotide chain to "(LNA-A)-succinate-CPG" using DNA phosphoramidites to obtain "5'-GCGAAAGGGGGATGTGCTGC(LNA-A)-succinate-CPG". In addition, a person of ordinary skill in the art would consider that Latorra et al. treated 5'-GCGAAAGGGGGATGTGCTGC(LNA-A)-succinate-CPG with a mild aqueous base. Finally, a person of ordinary skill in the art would consider that Latorra et al. would obtain LNA Forward primer 2, 5'- GCGAAAGGGGGATGTGCTGCA-3', containing the LNA residue at the 3'-end such as the following Forward primer 2: 5'-GCGAAAGGGGGATGTGCTGC(LNA-A) -3'.

As described above, a person of ordinary skill in the art would consider that Latorra et al. used primers containing a LNA at the 3' terminal position. Therefore, a person of ordinary skill in the art would not consider to place a LNA at the third position from the 3' end based on the disclosure of Latorra et al.

The above sentences on page 81, first paragraph in the left column of Latorra et al. are under the heading ""MATERIALS AND METHODS." This portion of Latorra et al. thus shows how to prepare materials for the experiments in the Latorra et al. article.

In other words, as explained above, the sentences "The 3' LNA residues were attached to controlled pore glass" and "The remaining sequences were made using standard DNA phosphoramidites produced by Proligo LLC." means that LNA is restricted to 3' end and other nucleotides are usual nucleotides (phosphoramidite). A nucleotide having a LNA as the third nucleotide from the 3' end of an oligonucleotide cannot be made by the method described in Latorra et al. The aforementioned sentences on page 81 of Latorra et al. teach only how to make primers which have LNA at the 3' end.

Therefore it is clear that there is no disclosure in Latorra et al. of a nucleotide having a LNA at the third nucleotide from the 3' end of the oligonucleotide.

5. The following was stated on page 3, the sixth paragraph in the ADVISORY ACTION dated December 5, 2008:

"Furthermore, the declaration does not provide evidence that the substituting an LNA or an ENA as third nucleotide from the 3' end results in any unexpected result."

The declaration referred to in the preceding paragraph is the DECLARATION UNDER 37 CFR 1.132 of Dr. Makoto KOIZUMI dated October 29, 2008 (hereinafter referred to as the "October 28, 2008 KOIZUMI DECLARATION").

As explained above, there is no teaching or suggestion in Latorra et al. concerning oligonucleotides having a LNA as third nucleotide from 3' end.

Substituting an ENA or an LNA as third nucleotide from the 3' end results in an unexpected result as explained as follows:

The advantage and the problems of Allele-specific PCR (AS-PCR) are described on page 79, lines 16 et seq., left column in Latorra et al. as follows:

"AS-PCR has the advantage of combining the amplification and detection events, with no additional probes or enzymes required. The technique has fallen from favor with natural DNA primers due to false priming of certain nucleotides leading to inaccurate genotyping as described in the literature for the past decade. With more prevalent transition SNPs, easily discriminated 3' primer:template junctions G:G or A:A cannot be used in allele-specific primer design. Significant levels of mismatch extension from G:T, C:A, A:C, and T:G combination has been observed."

Heretofore, a serious problem was that significant levels of mismatch extension from the 3' primer:template junction T:G is observed. As shown In Fig. 6 and 7 in the above-identified patent application, in the case of using DNA primers, mismatch extension from the 3' primer:template junction T:G was 67% of match extension from the 3' primer: template junction C:G. On the other hand, when a primer containing a LNA unit at the third position from the 3' end was used, the mismatch extension from the 3' primer:template junction T:G was 15% of match extension from the 3' primer: template junction C:G. Moreover, in the case of using ENA primers containing the ENA

unit at the third position from the 3' end, mismatch extension from the 3' primer:template junction T:G decreased to only 6% of match extension from 3' primer:template junction C:G.

The concern for improvement of AS-PCR is described in the sentence bridging the left and right columns on page 79 in Latorra et al. as follows:

> "Accurate detection of transition SNPs using allele-specific DNA primers requires an investment in primer design, optimization time, and reagent."

In terms of the desire for a more accurate detection for AS-PCR, ENA primers, with a lower percentage of mismatch extension than those of DNA and LNA primers, are clearly preferred in comparison with DNA and LNA primers.

It is respectfully submitted that a person of ordinary skill in the art would not consider substituting the ENA units of Koizumi et al. for the LNA units of Latorra et al. for the following reasons:

The following was stated in the seventh paragraph on page 2 in the December 5, 2008 ADVISORY ACTION:

> "The declaration [the October 29, 2008 KOIZUMI DECLARATION] further presents the arguments that Koizumi et al. do not teach a modified oligonucleotide used for a protein-DNA molecular interaction. However, the protein which Applicant cites is a polymerase and the modified oligonucleotide is a primer used to elongate/amplify DNA. However, Lattora et al. teach modified primer interactions/amplifications in Polymerase Chain Reaction (PCR, entire article)."

ENA units are considered to be superior to LNA units in triplex formation as described in Koizumi et al. This means that triplex forming oligonucleotides ("TFOs") containing ENA units can tightly bind to double-strand DNA to form triplexes compared

to TFOs containing LNA units. In Koizumi et al., molecular interaction between double-strand DNA and modified oligonucleotides such as ENA and LNA is discussed.

Molecular interaction between double-strand DNA and TFO is described in Chapter 27 on page 487, lines 1 et seq. of Applied Antisense Oligonucleotide Technology, edited by C.A. Stein and Arthur M. Krieg as follows:

> "Triple helix formation can be mediated by binding of selected oligonucleotides to homopurine regions of the duplex DNA. These triplex-forming oligonucleotides (TFO) bind specifically in the major groove of the DNA, forming hydrogen bonds with bases in purine-rich strands."

A copy of page 487 of Applied Antisense Oligonucleotide Technology was provided with the October 29, 2008 KOIZUMI DECLARATION.

However, in the above-identified patent application, I disclosed how DNA polymerase recognizes double-strand DNA with a template and a primer and elongates a strand. It is a molecular interaction between a protein (DNA polymerase) and a double-strand DNA modified with ENA.

Molecular interaction between protein and double-strand DNA is described in the Summary on page 345 of Nucleic Acids in Chemistry and Biology, edited by G. Michael Blackburn and Michael J. Gait as follows:

> "All nucleic acids have repeating polyanionic backbones, and so all proteins that bind to nucleic acids have strategically placed arginines and lysines that create an electrostatic field to neutralize the negative charge. To interact with B-DNA, the protein either (1) inserts an alpha-helix into the major groove, or (2) inserts a beta-sheet into the minor groove, and forms hydrogen-bonds from the side-chains to specific bases."

A copy of page 345 of Nucleic Acids in Chemistry and Biology was furnished with the October 29, 2008 KOIZUMI DECLARATION.

The disclosure in Koizumi et al. involving a molecular interaction between double-strand DNA and modified oligonucleotides such as ENA and LNA, is completely and qualitatively different from the above-identified application, which concerns a molecular interaction between a protein and a double-strand DNA modified with ENA. Therefore, if one of ordinary skill in the art reads Koizumi et al. and understands that ENA units are superior to LNA units in triplex formation involving a molecular interaction between double-strand DNA and modified oligonucleotides such as ENA and LNA, a person having ordinary skill in the art would not consider to substitute ENA units for LNA units for primers in a Polymerase Chain Reaction involving a molecular interaction between DNA polymerase and a double-strand DNA.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: Jan, 23, 2009

Makoto KOIZUMI

Nucleic Acids in Chemistry and Biology

Edited by
G. Michael Blackburn
and Michael J. Gait

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Contents

tries. Controlled pore glass beads (CPG) are ideal in being rigid and non-swellable. They are manufactured with different particle sizes and porosities, and they are chemically inert to reactions involved in oligonucleotide synthesis. Currently, 500 and 1000 Å porosities are favoured. The silylation reactions involved in functionalization of glass (introduction of reactive sites) are beyond the scope of this chapter. It is sufficient here to note that a long spacer is used to extend the sites away from the surface and ensure accessibility to all reagents. One type of spacer is illustrated (Fig. 3.59). The loading of amino groups on the glass is best kept within a narrow band of 10–50 μmol g^{-1}, below which the reactions become unreproducible and above which they are subject to steric crowding between chains.

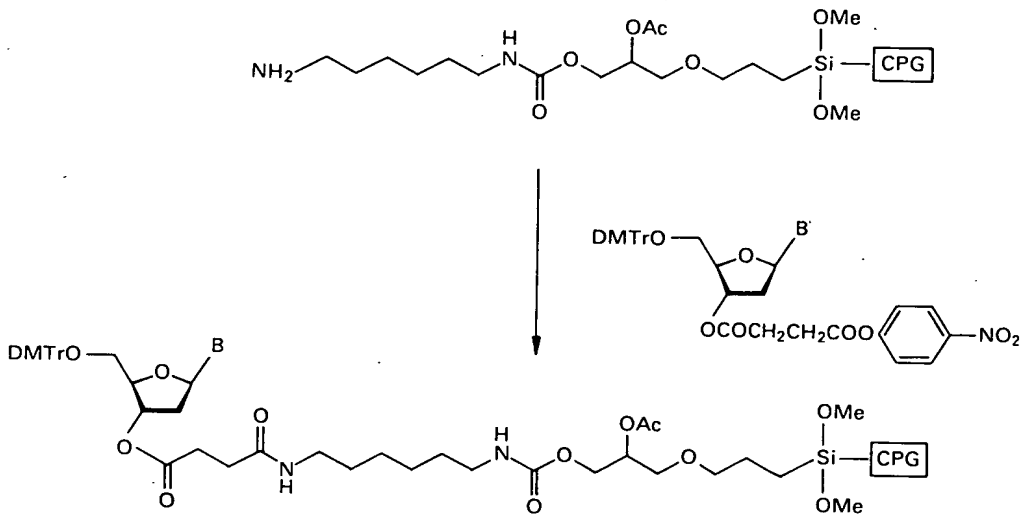

Fig. 3.59 Attachment of a nucleoside to a solid support of controlled pore glass (CPG) functionalized by a long chain alkylamine.

The 3′-terminal deoxynucleoside of the oligonucleotide to be synthesized is attached to the CPG support by conversion of its 5′-*O*-DMTr-(*N*-acylated)-derivative into the corresponding 3′-*O*-(4-nitrophenyl) succinate, which is subsequently reacted with amino groups on the support (Fig. 3.59).

Assembly of oligonucleotide chains

Assembly of the protected oligonucleotide chain is carried out by packing a small column of deoxynucleoside-loaded glass (as little as 10 mg can be used, 0.2 μmol) and flowing solvents and reagents through in predetermined order. This is most reproducibly accomplished using a commercial DNA synthesizer, but a manual flow system or even a small sintered glass funnel can be substituted. Machine specification

varies considerably, but the basic steps involved in one cycle of nucleotide addition using the popular phosphoramidite chemistry are shown (Fig. 3.60).

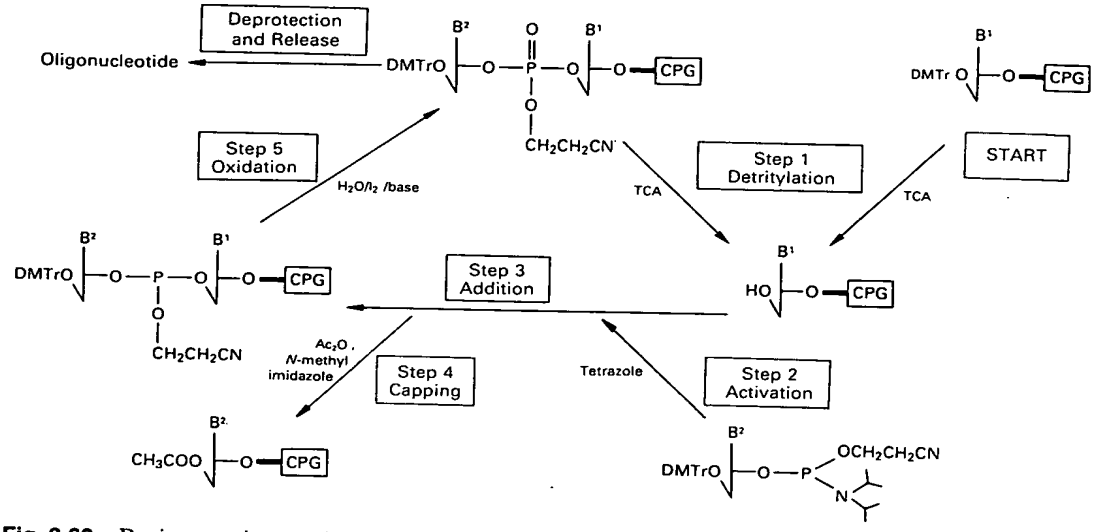

Fig. 3.60 Basic steps in a cycle of nucleotide addition by the phosphoramidite method.

Step 1. Detritylation (removal of dimethoxytrityl groups) is accomplished with dichloroacetic or trichloroacetic acid (TCA) in methylene chloride. The orange colour (dimethoxytrityl cation) liberated into solution is compared in intensity with the detritylation of the previous cycle to obtain the **coupling efficiency**.

Step 2. Activation of the phosphoramidite occurs when it is mixed with tetrazole in acetonitrile solution.

Step 3. Addition of activated phosphoramidite to the growing chain.

Step 4. Capping is a safety step introduced to block chains which are somehow not reacted during the coupling reaction and is designed to limit the number of failure sequences (those missing an internal residue). A fortuitous benefit of this step is that phosphitylation of the O–6 position of guanine is reversed.

Step 5. Oxidation of the intermediate phosphite to the phosphotriester is achieved with iodine and water. Pyridine or 2,6-lutidine is used to neutralize the hydrogen iodide produced.

The cycle is repeated the requisite number of times for the length of oligonucleotide required, with each deoxynucleotide phosphoramidite being added in the desired sequence and building from 3′-to-5′.

Deprotection and removal of oligonucleotide from the support

(i) The 5′-DMTr group is removed with the same detritylating agent as used in the assembly cycle.

(ii) The phosphate protecting groups are removed. In the phosphotriester method, an aryl protecting group is selectively displaced using *syn*-2-nitrobenzaldoximate ion or 2-pyridinecarbaldoximate ion. The product undergoes rapid elimination in the presence of water (see Fig. 3.29). In phosphoramidite synthesis, a methyl protecting group is removed with thiophenate ion (generated with thiophenol and triethylamine) which acts by nucleophilic attack on the methyl group, followed by hydrolysis. Alternatively, a 2-cyanoethyl group is removed by β-elimination using aqueous triethylamine or ammonia (Section 3.2.2).

(iii) All heterocyclic base protecting groups are removed with concentrated aqueous ammonia at room temperature for 24–48 h or at 50°C for 5 h.

(iv) The succinate linkage is cleaved with mild aqueous base.

In phosphoramidite chemistry using the 2-cyanoethyl group, steps (ii)–(iv) are carried out simultaneously using aqueous ammonia.

Purification of oligonucleotides

The importance of a good separation technique for synthetic oligonucleotides is often neglected. Since the impurities from a large number of reactions are stored up on the support (at least those pertaining to the growing chain), these must all be resolved, preferably in a single chromatographic step. Fortunately, powerful separation methods have been developed for purification of μg to mg quantities.

(i) **Polyacrylamide gel electrophoresis** separates oligonucleotides by virtue of their unit charge difference. This method is particularly useful for the purification of long oligonucleotides (>50 residues), but is limited to small scale (up to 1 mg). Single nucleotide resolution is possible to well beyond 100 residues.

(ii) **High performance liquid chromatography** is suitable for purification of perhaps up to 50 mg of oligonucleotide. Ion exchange chromatography resolves predominantly by charge difference and is useful both diagnostically and preparatively for oligonucleotides up to about 50 residues. Reversed phase chromatography separates according to hydrophobicity. Here the elution position of a fully deprotected oligonucleotide is hard to predict. More reliable identification of products is achieved by leaving the highly lipophilic 5′-DMTr group intact, such that the oligonucleotide is well resolved from shorter, non-DMTr containing impurities. The DMTr group is removed following chromatography.

Summary

The four essential steps in solid-phase synthesis are: (i) attachment of the first deoxynucleoside to the support, (ii) assembly of the oligonucleotide chain, (iii) deprotection and removal of the oligonucleotide from the support and (iv) purification. Assembly of the protected oligonucleotide chain can be carried out on a very small scale with the aid of a machine to add solvents and reagents reproducibly to the solid support, followed by filtration purification.